# Photodynamic action of the red laser on *Propionibacterium acnes*[*]

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**Abstract:** Background: Photodynamic therapy is a therapeutic modality that has consolidated its activity in the photooxidation of organic matter, which arises from the activity of reactive oxygen species.
Objective: To evaluate the effect of red laser 660nm with the photosensitizer methylene blue on *Propionibacterium acnes in vitro*.
Method: The experimental design was distributed into four groups (1 - control group without the application of light and without photosensitizer, 2 - application of light, 3 - methylene blue without light, and 4 - methylene blue with light). Tests were subjected to red laser irradiation 660nm by four cycles of 5 minutes at 3-minute intervals.
Results: It was evidenced the prominence of the fourth cycle (20 minutes) groups 2, 3 and 4.
Study limitations: Despite the favorable results, the laser irradiation time photosensitizer associated with methylene blue were not sufficient to to completely inhibit the proliferation of bacteria.
Conclusion: Further studies *in vitro* are recommended to enable the clinical application of this photosensitizer in photodynamic therapy.
**Keywords:** Laser therapy; Methylene blue; *Propionibacterium acnes*

## INTRODUCTION

Acne is an inflammatory disease of the pilosebaceous unit, characterized by mild lesions, from the comedogenic type, to severe lesions, which can cause large and unsightly scars.[1] One of the microorganisms involved in the inflammatory response of acne is *Propionibacterium acnes* (*P. acnes*), a Gram-positive bacterium, which alters the lipid composition of the sebum, causing an inflammatory medium.[2,3]

Clinically, it is classified into five levels: grade I (comedonic), with the presence of closed and open comedones; grade II (papular-pustular), in which comedones are associated with papules and pustules of purulent content; grade III (nodule-cystic), when more exuberant nodules are added; grade IV (conglobata), with formation of abscesses and fistulas; and grade V (fulminans), a rare form that causes a drop in the general condition of the patient and requires hospitalization.[4]

Currently, treatment of acne uses oral or topical drugs, alone or in association. However, there are still cases where these options cannot be used, either due to their adverse events or to the lack of response to the treatment observed with increasing bacterial resistance to antibiotics.[5]

In attempt to find new therapeutic options, photodynamic therapy (PDT) has been considered a promising treatment for acne, in addition to being considered a fast and effective method.[6]

PDT is a consolidated therapeutic modality that has its activity in the photo-oxidation of biological matter, resulting from the activity of reactive oxygen species (ROS) and singlet oxygen. Such species cause cell death by visible light in the presence of a photosensitizer and oxygen.[7]

In the photodynamic process, the photosensitizer (Ps), which is in the ground state, is activated by light, absorbing radiant energy, passing into the excited singlet state. After the excitation, Ps leads to the triplet state, which has the longest lifetime in relation to the excited singlet state. In the triplet state, Ps has a lifetime on the microsecond scale, sufficient to generate ROS. These species can be generated by abstraction or absorption of electrons or hydrogens, leading to the formation of free radicals, which induce cell death. Also by deactivating the triplet state by transferring energy to molecular oxygen, there is formation of singlet oxygen, a mechanism that also leads the cell to death. Both "ROS" and "singlet oxygen" are mechanisms that can lead biological matter to apoptosis.[7,8]

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PDT mechanisms involved in the treatment of acne include photodamage of *P. acnes*, as well as reduction of size of sebaceous glands and also decrease of production of sebum.[9]

Methylene blue (MB) photosensitizer is derived from a molecule belonging to the class of phenothiazines, classical for its important role in microbiology and pharmacology as a cellular marker and clinical use in the treatment of meta-hemoglobinemia.[10]

The objective of the study was to evaluate the action of 660 nm red laser with the methylene blue photosensitizer on *Propionibacterium acnes in vitro*.

## METHODS

**Biological Material:** *Propionibacterium acnes* strain ATCC (American Type Culture Collection) 6919 was used, which was cultured for 24 hours on Trypticase Soy Agar (TSA, OXOID®) medium, using incubation at 35° C.

**Photosensitizer:** The MB 1% photosensitizer was assayed, dissolved in dimethyl sulfoxide (DMSO), at a concentration of 1mg/mL$^{-1}$, and then diluted in NaCl solution (0.5%) to obtain 20μM concentration of the photosensitizer solution.

**Light source:** The LASERLine 110V red laser diode was used, with a wavelength of 660 nm, with energy density 192 J/cm$^2$ and continuous laser beam emission.

**In vitro Assay:** *P. acnes* ATCC 6919 was cultured for 24 hours in Tripticase Soy Broth, (TSB, Oxoid®) medium, when centrifugation (3000 rpm) was performed for one minute, then supernatant was discarded, and the precipitated material was resuspended in sterile NaCl solution (0.5%) and again subjected to centrifugation. This procedure was repeated five times for washing the bacterial cells and removing components from the culture medium. The precipitated material obtained was resuspended in 0.5 mL of sterile NaCl solution (0.5%) and stirred vigorously for one minute, then 4.5mL of NaCl solution was added, and the solution was stirred for one minute, being adjusted to 0.5 Mc Farland standard tube, which corresponds to approximately 1.5 x $10^8$ mL$^{-1}$ colony forming unit (CFU). From this solution, serial dilutions were performed resulting in a concentration of 1.5 x $10^5$ CFU mL$^{-1}$.

**Anti-bacterial activity:** Photodynamic action was performed using "completely randomized design" study type, consisting of four groups: G1 - without light application and without photosensitizer (control group); G2 - application of light on the bacteria; G3 - bacteria with MB without light irradiation; and G4 - bacteria with MB with light. Experiment was conducted under in vitro conditions in November and December 2014. 1.05 mL of the bacterial suspension and 0.05 mL of the photosensitizer solution were deposited in the test tubes. Assays were held under light and 35 °C for 15 minutes for pre-irradiation time and then subjected to irradiation for four five-minute cycles with three-minute intervals. Samples were irradiated with red laser with wavelength of 660 nm. At each cycle, an aliquot of 0.05 ml was removed and transferred to the Petri dish containing TSA agar. In the medium, it was uniformly distributed with the aid of Drigalski's handle, and the plates were incubated at 35 °C for 24 hours. After this period, CFU count was evaluated for the photodynamic activity on *P. acnes*. The whole procedure was performed in triplicate.

**Statistical analysis:** The 48 samples collected were analyzed for distribution using Shapiro-Wilk and D'Agostino & Pearson tests, showing distribution close to normality. Statistical treatment for comparison of different therapeutic times within the same group was performed using non-parametric one-way ANOVA with Tukey's post-hoc test, adopting a 95% confidence interval ($p<0.05$). Each group was analyzed in isolation. For comparative analysis between groups, two-way ANOVA test for non-repeated measures and Bonferroni post-hoc test for comparison of groups were used. Confidence interval adopted was 95% ($p<0.05$).

## RESULTS

Table 1 shows the comparison of means of number of colonies obtained after incubation of samples in relation to different groups and different experimental times. Mean is related to standard deviation, demonstrating the probable deviation of each group in relation to samples tested. Legends (a) and (b) represent the times within a same group that showed significant difference between them.

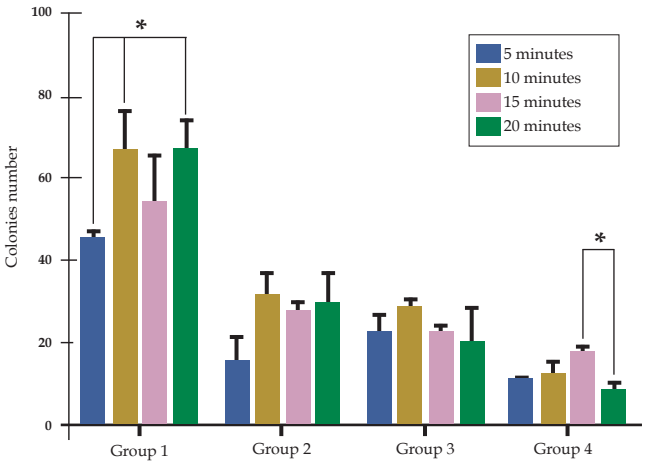
Graph 1 represents the number of colonies obtained for each of the four groups relating the treatment times within the same group. In Group 1, there was a significant difference only between 5 and 10 minutes and between 5 and 20 minutes. In Group 4, there was a difference between 15 and 20 minutes. There was no difference between exposure times in Groups 2 and 3.

Graph 2 represents the comparison between the different experimental times and results obtained in the number of colonies for each of the four groups. In the five-minute treatment, there was a significant difference between Group 1 and Groups 2 (a), 3 (b) and 4 (c). In the 10-minute treatment, there were differences between Group 1 and Groups 2 (a), 3 (b) and 4 (c), and there was also a difference between Group 2 and Group 4 (d) and between Group 3 and Group 4 (e). In 15 minutes, there was difference between Group 1

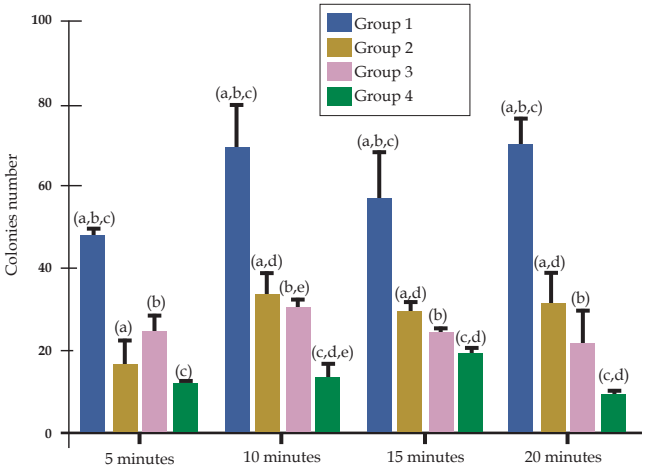
**Table 1. Comparison of the mean ± standard deviation of the number of colonies obtained after different experimental times. (a) and (b) represent the difference between the experimental times within the same group**

| | 5 minutes | 10 minutes | 15 minutes | 20 minutes | *p*-value |
|---|---|---|---|---|---|
| GROUP 1 | 46.00±2.64(a,b) | 67.00±16.82(a) | 54.50±16.26 | 67.33±11.59(b) | 0.020 |
| GROUP 2 | 16.00±8.48 | 32.00±9.16 | 28.00±4.36 | 30.00±12.77 | 0 |
| GROUP 3 | 23.00±7.55 | 29.00±2.82 | 23.00±2.64 | 20.50±12.02 | 0.656 |
| GROUP 4 | 11.50±0.70 | 12.50±4.95 | 18.33±2.08(a) | 8.66±3.78(a) | 0.050 |

Group 1 - without light application and without photosensitizer (control group); Group 2 - application of light on the bacteria; Group 3 - methylene blue (MB) without light irradiation; and Group 4 - MB with light




**Graph 1 :** Analysis of different experimental times within the same group. There was a statistically significant difference between the total number of colonies and the experimental times represented by the legend (*), p <0.05




**Graph 2 :** Relationship between the total number of colonies and the different sample times for each group. In the comparison between groups for each experimental time, there was a statistically significant difference between groups (a), (b), (c), (d) and (e), p <0.05

and Groups 2 (a), 3 (b) and 4 (c), and difference between Group 2 and Group 4 (d). And, in 20 minutes, there was difference between Group 1 and Groups 2 (a), 3 (b) and 4 (c) and between Group 2 and Group 4 (d).

## DISCUSSION

According to the analysis of the data obtained, Group 1 (control) showed a tendency to the growth of microorganisms in 10, 15 and 20 minutes, in which values of the number of colonies remained close and balanced. It reflects the natural growth of the colonies without any external intervention. When analyzing graph 2, it can be seen that Group 1 was significantly higher in comparison to Groups 2, 3 and 4, in all sample times.

Groups 2 and 3 behaved in a similar manner, showing an initial reduction in the number of colonies of the microorganism. However, this reduction did not extend after five-minute phototherapy, with colony growth at a level close to that of five minutes at all sampling times. Thus, there was no significant difference between different times for both groups, as verified in graph 1. When analyzing the sample times comparatively between the four groups, present in graph 2, it can be seen that Groups 2 and 3 have colonies counting significantly lower than those presented in Group 1. Also, Groups 2 and 3 presented similar efficiency in controlling microorganism populations.

Group 4 presented the treatment with the best comparative values, as observed in graph 2. Number of colonies at all sample times in this group was lower than in the other three groups. Emphasis was given to 10, 15 and 20 minutes, where the number of colonies was significantly lower in relation to Groups 1, 2 and 3. When analyzing graph 1 regarding different times of phototherapy associated with therapy proposed to Group 4, it was observed that a more prolonged therapy was not more efficient for this treatment, presenting only difference between 15 and 20 minutes. It is inferred that, from the first five minutes of therapy, there is an inhibition in the growth of microorganism colonies, and this inhibition remains for the next 20 minutes and is more efficient when compared with groups 1, 2 and 3.

Photodynamic action of methylene blue photosensitizer and red laser was studied in the inhibition of *P. acnes*, in which a comparative study of CFUs was conducted. In the experiment, *P. acnes* was inhibited by the photodynamic action, presenting photosensitization of methylene blue when irradiated with red laser with a wavelength of 660 nm, being presented in graph 1.

As it is well known, the tested groups used elements such as methylene blue photosensitizer and red light on the bacteria, important elements for the action of PDT. It should be emphasized that these PDT elements enter into action, leading bacterium to oxidative stress, due to the formation of singlet oxygen and reactive oxygen species, and then to cell death.[7,8]

Results demonstrated that application of red laser inhibits the growth of CFUs of bacteria. These results corroborate the findings of Ogata and Fernandes, who used the same phototherapy as acne treatment *in vitro*; however, treatment times used by the authors were different, being 5, 15, 30 and 60 minutes. Their results were excellent, with zero CFU of *P. acnes* bacterium.[11]

In the test using only light, a reduction of CFUs was observed. This activity had a positive evolution for the four cycles of G2, evidencing that the light reached the plateau without reaching the total elimination of the *P. acnes* CFUs (Table 1). For Ashkenazi et al., this limitation can be attributed to a possible saturation of the membrane, because the effect of light on *P. acnes* is due to the bacteria synthesizing porphyrins, mainly protoporphyrin and coproporphyrin, photosensitive substances that, when receiving light, absorb its energy, and this energy interferes with cellular chemical and metabolic reactions, and when light is absorbed by porphyrins, ROS formation occurs, resulting in damage to the bacterial membrane and cell death.[12]

However, when light is associated with Ps (group G4 - Table 1), it shows elimination of bacteria by photoinduced process, since

this process is initiated by transfer of hydrogens or electrons, radical reactions or by electrophilic attack of singlet oxygen to membranes, leading to apoptosis of biological matter.[13,14] This process demonstrates the incorporation of MB on *P. acnes*, as the photoinduced process led to the elimination of the bacterium.

Incorporation of MB on *P. acnes* leads to the optimization of the photo-oxidative process. Thus, with the knowledge that the photosensitizer can be found both on cell walls and in organelles, action of PDT can occur in a generalized way, propagating both by internal attack (intracellular medium) and external attack (extracellular medium), which justifies the partial inactivation of *P. acnes* (G3 - Table 1).

Therefore, for a more detailed study on photosensitizer incorporation, the study indicates the need for a fluorescence microscopy work to analyze the incorporation of the photosensitizer into *P. acnes*.

## CONCLUSION

Red laser and MB photosensitizer, applied separately on *P. acnes*, were not effective for total inhibition of bacterial colonies, only promoting a partial reduction of them; but when associated, they were efficient, being the best treatment *in vitro* compared with the other groups. Further *in vitro* research is suggested, increasing the time of assay and other types of photosensitizers and also the use of fluorescence microscopy to study the action of PDT for the treatment of *P. acnes*. ❑

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